An Improved Measurement of Progesterone in Saliva and Clinical Applications*

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ABSTRACT

Measurement of progesterone in saliva offers several advantages when compared to assays of serum progesterone, especially when ovarian activity is being assessed. Most published methods for the determination of progesterone in saliva are based on assays developed in research centers, which employ "in-house" reagents that are critically dependent on supplies of highly selected antisera. In this report, the adaptation of a readily available commercial progesterone "kit", the Pantex Immunodirect Progesterone (125I) is described for the measurement of salivary progesterone. A single extraction step was added, however, to improve assay performance and to ensure that total salivary progesterone was measured.

Introduction

Progesterone levels can be measured by modern immunoassay techniques employing radiolabelled tracers as well as by enzyme-linked colorimetric methods or fluorimetric techniques. These progesterone assays provide an objective method for evaluation of ovarian function. During the evaluation of female infertility, serum progesterone levels are commonly measured once per cycle. Daily blood sampling is poorly tolerated by patients, although serial determinations might provide a more accurate picture of luteal integrity than single determinations. In contrast, saliva is a readily available fluid in which the assay of steroid hormone levels has become established within the last five years.10 Several groups of investigators have developed "in-house" salivary progesterone radioimmunoassays (RIA).1,2 3,4,6,9,10,11,12,14,17,19,20,21,22,23,24,25 The adaptation of commercial "kits" for the same purpose offers convenience and would allow many laboratories to study infertile patients with ease.

Our experience is reported with a salivary progesterone assay using a modifi-
cation of a direct serum progesterone "kit", the Pantex Immunodirect Progesterone (125I) Kit. This assay has facilitated objective evaluation of many infertile women seen at the Emory University Clinic.

Materials and Methods

Reagents

Immunodirect Progesterone (125I) RIA kits were obtained.* The kits included (125I) progesterone-3-carboxymethylxime histamine (specific activity 2000 Ci per mmole), progesterone zero standard, and progesterone standards in human serum with concentrations of 0.2, 0.5, 2.5, 5, 10, 20, 40 and 80 ng per ml. It also included first antiserum raised to progesterone 11-α-hemisuccinyl-BSA conjugates in rabbits and precipitating antirabbit antiserum which was produced in goats. General assay buffer (PBS) contained 0.008 M disodium phosphate, 0.002 M potassium phosphate, 0.140 M sodium chloride, and 0.003 M potassium chloride and had a pH of 7.4. 3H-progesterone 1,2,6,7(N) (specific activity 90 Ci per mmole) was obtained.† It was diluted on receipt with ethanol 1/20 and stored at 4°C for not longer than four months. Progesterone,‡ from steroid-stripped human serum,§ and hexane‖ (b.p.68-70°) of spectrophotometric grade was also obtained.

Progesterone-free saliva was prepared as follows: 1,2,6,7-3H progesterone (10 × 10⁶ cpm) was equilibrated with pooled male saliva at 22°C for 30 min. Seven grams of washed alkaline Norit A charcoal¶ was used to strip 100 ml saliva. After agitating the mixture for 24 to 36 hrs at 4°C and centrifuging at 3000 g for 30 min, the charcoal pellet was separated. Two hundred fifty mg laboratory-grade Kaolin‖ per three ml of saliva were added and the mixture agitated for one to two mins. After centrifugation at 3000 g for 20 min, an aliquot of the treated saliva showed negligible amounts of radioactivity.

Sample Collection

Subjects were asked to expectorate five ml of saliva into polypropylene tubes every morning between 0600 and 1000 hr, 15 min after the mouth was rinsed with water. Specimens were capped and stored in the home freezer until they could be brought to the laboratory where they were thawed and centrifuged at 1500 g for 15 min. The supernatant was stored in polypropylene tubes at −20°C or lower until assayed.

Serum Collection

Blood was obtained by venipuncture using either plain glass or serum separator tubes.*

Quality Control Materials

Four quality control saliva pools representing different concentrations of progesterone were used to assess intra- and inter-assay variation. The zero pool was composed of charcoal-stripped male saliva. The low pool consisted of female saliva from the follicular phase. The medium and high pools consisted of saliva from pregnant volunteers diluted if necessary with charcoal-stripped saliva.

* Pantex Division of Bioanalysis Inc., Santa Monica, CA.
† New England Nuclear, Boston, MA.
‡ Sigma Chemical Co., St. Louis, MO.
§ Chemicon International Inc., Los Angeles, CA.
¶ Mallinckrodt, Inc., Paris, KY.
‖ Fisher Scientific, Pittsburgh, PA.
¶¶ Fisher Scientific, Pittsburgh, PA.
* Becton Dickinson Vacutainer Systems, Rutherford, NJ.
ASSAY OF PROGESTERONE IN SERUM

The assay in serum was a direct assay (no extraction), performed as described by the manufacturer. Briefly, 100 μl of serum or standard including the zero standard was incubated (37°C, one hr) with 100 μl first antiserum and 500 μl \(^{125}\)I-progesterone derivative. The antibody-bound progesterone was precipitated with 500 μl second antiserum during a 10 min incubation at room temperature followed by centrifugation at 2500 g for 15 min. The supernatant was discarded and the pellet counted.

ASSAY OF PROGESTERONE IN SALIVA

Progesterone was extracted from whole saliva (one ml) with hexane (8 ml). The optimum extraction procedure was attained when the tubes were shaken for a five min period using a multivortex shaker. The aqueous phase was frozen by immersing the tubes in a solid CO\(_2\) and ethanol mixture and the organic phase decanted into polypropylene tubes suitable for radioimmunoassay. The solvent was evaporated under nitrogen at 45°C. The extraction was carried in duplicate samples and to each sample 10 μl of steroid-free serum was added followed by 90 μl of assay buffer.

For the standard curve, 100 μl of the standards having been further diluted (1:9, v/v) with assay buffer provided standards that were 10 percent of those used for the serum assay (namely 0, 0.02, 0.05, 0.25, 1, 2, 4, and 8 ng per ml). Standards were assayed in duplicates. To the same volume of sample (or standard) as used in the serum assay (100 μl) was added 100 μl of \(^{125}\)I progesterone (6000 cpm, 8 pg, namely one-fifth of that used in the serum assay) followed by 100 μl of antiserum having been pre-diluted with assay buffer (1:4, v/v, one fifth of that used in the serum assay). The incubation time was three hr at 37°C in a water-bath, and the bound and free fractions separated by the addition of 100 μl of second antiserum, one hr incubation at 22°C and centrifugation at 2500 g for 20 min. At this concentration of reagents and under these conditions, 50 percent of eight pg of iodinated progesterone was bound. The tubes containing the (antibody-bound) pellet were counted on a Multi-Prias gamma counter, equipped with a Model A504.* Data reduction was performed by the Corning Inform System.† The dose-response curve was obtained and concentrations of progesterone in the samples were calculated by using a logit-lot fit.\(^{16}\)

RECOVERY OF PROGESTERONE FROM SALIVA

An ethanolic solution of 1,2,6,7-\(^{3}\)H-progesterone was used. Aliquots (0.1 ml, 6000 cpm) were added to separate culture tubes and the solvent evaporated. One ml of saliva was added to each tube and the mixture allowed to equilibrate for 30 min at room temperature. Extraction with hexane was performed as previously described. Aliquots of the extracted material were added to four ml liquid scintillation fluid: and the samples counted for 10 min each on a Beckman liquid scintillation counter.

PROTEIN DETERMINATION

Protein concentrations in saliva samples were determined by the method of Lowry et al.\(^{8}\)

STATISTICAL ANALYSIS

Standard definitions were used for range, mean, standard deviation (S.D.)

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* Hewlett-Packard Co., Palo Alto, CA.
† Corning Medical and Scientific, Medfield, MA.
‡ Maxifluor; J. T. Baker Chemical Co., Phillipsburg, NJ.
and coefficient of variation (C.V.). The linear regression coefficient \( r \) was calculated for values of progesterone in paired saliva and serum samples and for corresponding protein and progesterone concentrations. A \( p \) value less than 0.05 was considered significant.

**Subjects**

Eight healthy female volunteers with regular menstrual cycles who had not taken oral contraceptives for at least six months were asked to provide saliva samples every day for one or two menstrual cycles, during which they recorded basal body temperature (BBT) readings. Matched serum and saliva samples were obtained from six subjects. Seven subjects with documented biphasic temperature graphs were considered to have ovulated, and their data was used for calculation of normal salivary progesterone values. The temperature charts were used to divide the cycles into follicular and luteal phases.

Three pregnant volunteers provided saliva samples several times a week during various stages of pregnancy. One lactating woman provided saliva samples several times a week for four months, beginning six months after parturition.

Ten women undergoing investigation and treatment of infertility of at least one year's duration were asked to provide daily saliva samples. Five women underwent therapy with clomiphene citrate and progesterone, and three women were treated with human menopausal gonadotropins (HMG) during the study. The BBT charts and results of endometrial biopsies, hysterosalpingograms, laparoscopies and ultrasound studies were available for some patients.

This study was approved by the Human Investigations Committee of Emory University. Informed consent was obtained from all patients.

**Results**

**Standard Curve**

In figure 1 are shown composite response curves. Replicate assays (\( n = 12 \)) showed that precision of the standard curve varied from 4.1 to 9.2 percent. The mean value for ED50, defined as the standard concentration equivalent to 0.5 \( B_{\text{max}} \) (\( B_{\text{max}} \) = maximum binding) was 1.1 ± 0.11 ng per ml (3.5 ± 0.34 nmole per liter) for 12 replicate standard curves. Logit log transformation of the standard curve (figure 1) shows a linear response between 0.02 and 4 ng per ml.

**Precision**

When the quality control pools were repeatedly assayed in one run, the intra-assay C.V. shown in table I were obtained. When these pools were included in 10 routine assay runs, the inter-assay C.V. shown in table II were obtained. The imprecision in the region of the low pools was largely due to curve fitting and data reduction. The within-run C.V. for the radioactivity counts for the zero and low pools were eight percent (\( n = 6 \)) and six percent (\( n = 8 \)), respectively.

![Figure 1](image-url)
TABLE I
Intra-assay Coefficients of Variation in Quality Control Saliva Pools

<table>
<thead>
<tr>
<th></th>
<th>Zero Pool</th>
<th>Low Pool</th>
<th>Medium Pool</th>
<th>High Pool</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean*</td>
<td>3.3</td>
<td>136.1</td>
<td>330</td>
<td>865.7</td>
</tr>
<tr>
<td>S.D.</td>
<td>0.8</td>
<td>19.1</td>
<td>32.1</td>
<td>75.9</td>
</tr>
<tr>
<td>n</td>
<td>6</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>C.V. (%)</td>
<td>24.5</td>
<td>14.1</td>
<td>10</td>
<td>9</td>
</tr>
</tbody>
</table>

*Values in pg per ml.

RECOVERY AFTER EXTRACTION

Mean recovery of progesterone after hexane extraction was 88.9 ± 6.7 percent (n = 6). Results were corrected for recovery and routine monitoring of recovery was performed.

INTERFERENCES

The synthetic steroid medroxyprogesterone acetate* was added to male stripped saliva which was then assayed for progesterone. No cross-reactivity was observed (data not shown). Similarly, human menopausal gonadotropins (HMG)† did not cross-react. Protein concentration in saliva samples showed no correlation with progesterone levels (r₁₈ = -0.1587, p > 0.1).

SENSITIVITY

The sensitivity of the assay, defined as the quantity of unlabeled hormone required to inhibit binding of the tracer by an amount equal to two standard deviations compared with the inhibition in the absence of unlabelled hormone, was 42 pmole per liter.

DURING THE FOLLICULAR PHASE, MOST SALIVARY PROGESTERONE VALUES WERE LESS THAN 150 pmole per liter. Occasional higher values were noted (figure 2). During the luteal phase, large peaks in salivary progesterone were observed 4 to 11 days before menses in all cycles studied (figure 2, A and B). Luteal values ranged from <150 to 1189 pmole per liter. Because of this wide variation and the presence of peaks, calculation of a mean luteal value using all luteal phase peak values was considered meaningless. Instead, the single peak luteal progesterone levels in each cycle studied were examined; peak levels ranged from 393 to 1189 pmole per liter with a mean peak value of 787 ± 246 pmole per liter. Salivary progesterone graphs and BBT graphs showed biphasic patterns. In the follicular phase, both parameters described gave flat lines. However, luteal progesterone values showed a peaking pattern while the BBT plots showed relatively flat lines. Day-to-day variation of BBT did not correlate with day-to-day variation of salivary progesterone. Moderate fluctuations in day-to-day salivary progesterone levels resulted in small spikes during both the follicular and luteal phases (figure 2B). This spiking effect was present in six follicular

TABLE II
Inter-assay Coefficients of Variation in Quality Control Saliva Pools

<table>
<thead>
<tr>
<th></th>
<th>Low Pool</th>
<th>Medium Pool</th>
<th>High Pool</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean*</td>
<td>147</td>
<td>331.5</td>
<td>802.4</td>
</tr>
<tr>
<td>S.D.</td>
<td>25.6</td>
<td>57</td>
<td>113.5</td>
</tr>
<tr>
<td>n</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>C.V. (%)</td>
<td>17</td>
<td>17.2</td>
<td>14</td>
</tr>
</tbody>
</table>

*Values in pg per ml.

* Provera; The Upjohn Company, Kalamazoo, MI.
† Pergonal; Serono Laboratories, Inc., Randolph, MA.
Salivary progesterone levels ranged from 765 pmole per liter at the sixth week of gestation to 4748 pmole per liter at the 34th week (figure 3A). There was marked variation in salivary progesterone levels between individual pregnant women and from day to day in the same pregnant woman. Progesterone levels in early and mid-pregnancy overlapped with luteal phase values. A dip to 543 pmole per liter at week 11 was noted in a single patient, corresponding to the involution of the corpus luteum.

Salivary progesterone levels in the patient with a history of habitual abortion overlapped those of the normal luteal phase and those of normal preg-

Figure 2. Salivary progesterone levels for four normal women throughout the menstrual cycle. All subjects show a large luteal peak in the second half of the cycle. A. There is no day-to-day variation during the follicular phase but moderate variation is present in the luteal phase. B. In these two cases there is marked day-to-day variation in salivary progesterone in both follicular and luteal phases.

Figure 3. A. Salivary progesterone levels during pregnancy in three normal subjects. B. Salivary progesterone levels during early pregnancy in a patient with a history of recurrent abortion treated with progesterone at four months' gestation.
nant women in the first and second trimester (figure 3B). Marked variation was present before and during progesterone therapy.

**Salivary Progesterone Levels During Lactation**

Salivary progesterone was <170 pmole per liter during lactation (results not shown).

**Salivary Progesterone Levels in Infertile Patients**

Results from two patients subsequently found to have obstruction of the reproductive tract (endometriosis and cervical stenosis) were similar to those in normal volunteers (results not shown).

Results from one of three patients undergoing therapy with clomiphene citrate and progesterone suppositories are shown in figure 4. As expected, salivary progesterone levels were extremely high during progesterone therapy. Marked variation in progesterone levels was observed during therapy.

Results from three anovulatory patients undergoing therapy with HMG are shown in figure 5. One patient showed a progesterone peak 2.5 weeks after therapy (figure 5A). Another patient showed a well-developed progesterone peak beginning six days after a single dose of HMG and two and four days after two doses of human chorionic gonadotropin (HCG) (figure 5B). This peak was similar in height and breadth to those seen in normal luteal phases (compare figures 5B and 2). Multiple follicles were noted by ultrasonography of this patient. A third patient with premature ovarian failure showed a brief two-day progesterone spike one day after HMG therapy was begun (figure 5C).

**Comparison of Progesterone Concentrations in Serum and Saliva**

The correlation between salivary and serum progesterone levels was highly significant ($r_{36} = 0.93$, $p < 0.001$) and is shown in figure 6. The regression slope indicated that over the whole range, serum salivary progesterone represented 2.7 percent of progesterone found in serum.

**Discussion**

A commercially available "kit" has been modified for the measurement of progesterone in saliva at the pg level. Concentrations of reagents were decreased in order to increase the sensitivity of the assay. Most previously described methods have used tritiated tracers; radioiodinated tracers (this report) are more practical for the general laboratory where a gamma counter is the only counter available. Because the present authors found that results from the direct assay procedure given by the manufacturer were not reproducible in saliva, an extraction step was added. Although steroids in saliva have often been assumed to represent the free plasma fraction, recent work indicates that progesterone is significantly bound to macromolecules in saliva and for this reason extraction assays are recom-
selective extracting and tritiated progesterone was added to monitor recovery. Hexane was chosen for our work because it is easier to handle technically, and it is also less polar than ether and, therefore, extracts fewer additional interfering substances.

Absolute values for salivary progesterone in normal women found in this investigation are in the range of most other published values, although two groups have found 2-fold to 4-fold higher values during both the follicular and luteal phases. Well-defined luteal progesterone peaks were seen in all normal cycles, and this finding suggests that daily salivary progesterone levels may provide another modality for assessment of luteal function which is as good as that afforded by a single serum level combined with a BBT chart. Unlike the BBT chart, however, salivary progesterone patterns do not seem to be affected by incidental infections or inability to sleep. Such salivary testing might also decrease the need for endometrial biopsies, a painful procedure requiring a clinic visit. Daily saliva sampling is feasible because saliva is easy and cheap to collect and because dilution of reagents allows five times as many saliva tests as serum tests to be performed using the contents of a single kit.

Figure 5. Salivary progesterone levels in patients undergoing therapy with human menopausal gonadotropins. A. A peak is seen approximately 2.5 weeks after initiation of therapy. B. A well-developed progesterone peak lasting several days and similar to those seen in normal luteal phases is present shortly after initiation of therapy. Compare figure 2C. C. A brief two-day spike is present during therapy.

Figure 6. Correlation between salivary and serum progesterone, as measured during menstrual cycles using the same reagents.
Although it may appear that there is a wide range and, therefore, an overlap in the mean salivary progesterone levels during both the proliferative and the luteal phase, it should be noted that these values include the levels at the extreme ends of these phases, such as the day of ovulation, the day after ovulation, and just before ovulation and decrease just prior to the onset of menstrual flow. If the mean levels of salivary progesterone from midproliferative and midluteal phases were taken, no significant overlap in the serum would have been observed.

The possibility that a sample contains interfering substances is of special concern when analyzing saliva. The marked day-to-day variation which was noted in several women in both phases of the menstrual cycle and during pregnancy showed no relationship to salivary protein concentration and was present before and after extraction. It was not related to the time of day the sample was collected or the fasting or non-fasting state of the subject. Because other workers using several different assay procedures have noted such variation and because in this study excellent correlation was obtained between progesterone levels in blood and saliva, it is suggested by us that these marked variations in salivary progesterone may well reflect changes in circulating steroid. However, the possibility that these changes represent specimen contamination cannot be completely ruled out.

Salivary progesterone patterns in infertile women reflected their diagnoses obtained by other means. Women with physical obstruction of the reproductive tract had normal salivary progesterone patterns. Anovulatory patients had salivary progesterone levels approximating the sensitivity of the test while not on therapy; after HMG treatment, several different patterns were noted. In one patient, the presence of a broad progesterone peak 2.5 weeks after therapy suggested that ovulation had been induced by the drug. In a second patient, a well-developed progesterone peak similar to those of normal luteal phases occurred six days after a single dose of HMG and two and four days after two doses of HMG. Presumably, follicles had begun development before administration of HMG, and ovulation occurred around the time of initiation of therapy in this patient. In a third patient with premature ovarian failure, the occurrence of a narrow salivary progesterone spike remains unexplained. This spike was not broad enough to indicate the presence of a normally-functioning corpus luteum.

Our modified kit procedure detected exogenous progesterone given to patients in standard doses. Again, marked day-to-day variation was noted, and this may reflect variable absorption of the drug. Salivary assays may provide a means for monitoring dose-effect relationships in such cases. It was not possible for us to detect medroxyprogesterone acetate or HMG in vivo or in vitro, and this assay can therefore be used to follow luteal function in patients taking these medications.

In conclusion, salivary progesterone assays may be used to differentiate normal and abnormal menstrual cycles and may be useful in following infertility patients undergoing induction of ovulation. The marked day-to-day variation in normal and abnormal cycles and during pregnancy is as yet unexplained but may reflect the physiology of progesterone secretion; further work is needed to elucidate the genesis of this variation. As cost-control becomes more important, the ease of collecting saliva specimens without the use of ancillary venipuncture personnel suggests that similar assays may be more widely used in the future.

Acknowledgments

The assistance of the many patients and volunteers who provided saliva samples is gratefully acknowledged.
Reference

13. Package insert for immuno-direct progesterone 125I kit. Pantex Division of Bioanalysis Inc., Santa Monica, CA.